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Cloning of two clip-domain serine protease genes and their expression in response to exogenous hormone and immune challenge in *Lasioderma serricorne* (Coleoptera: Anobiidae)

CHEN Chun-Xu[1,2], XU Kang-Kang[1,2], YANG Hong[1,3,*], ZHAO Feng[1], YAN Yi[1,2], HU Da-Ming[4], YANG Wen-Jia[1,2]

(1. Provincial Key Laboratory for Agricultural Pest Management of Mountainous Regions, Institute of Entomology, Guizhou University, Guiyang 550025, China; 2. Guizhou Provincial Key Laboratory for Rare Animal and Economic Insect of the Mountainous Region, College of Biology and Environmental Engineering, Guiyang University, Guiyang 550005, China; 3. College of Tobacco Science, Guizhou University, Guiyang 550025, China; 4. China Tobacco Guizhou Industrial Co., LTD, Guiyang 550005, China)

Abstract:【Aim】As essential components of extracellular signaling cascades, clip-domain serine proteases (CLIPs) play important roles in insect development and innate immunity. This study aims to clone two CLIP genes from the cigarette beetle, *Lasioderma serricorne*, to clarify their developmental stage- and tissue-specific expression profiles, and to analyze their expression changes in response to exogenous hormone 20-hydroxyecdysone (20E) and immune challenge, so as to lay the foundation for further understanding the physiological functions of CLIPs in *L. serricorne*.【Methods】The full-length cDNAs of two CLIP-encoding genes (*LsCLIP*1 and *LsCLIP*2) were cloned using RT-PCR, and the deduced protein structure was predicted using different bioinformatics software, and the phylogenetic tree of insect CLIPs was constructed using the MEGA 6.06 program. The mRNA expression profiles of these two genes in different developmental stages [early larva (<24 h post egg hatching), late larva (older than the 4th instar), pupa (>48 h post pupation), early adult (<24 h post eclosion), and late adult (7 d post eclosion)] and different tissues (cuticle, fat body, gut, and carcass) of the 5th instar larvae, and the 4th instar larvae of *L. serricorne* injected with 20E (120 ng/larva) and peptidoglycan (0.2 μL) from *Escherichia coli* or *Staphylococcus aureus* were detected by quantitative real-time PCR (qPCR).【Results】The full-length cDNA sequences of *LsCLIP*1 and *LsCLIP*2 were cloned from *L. serricorne*, and both genes have an open reading frame of 1 194 bp encoding 397 amino acid residues. The predicted CLIP1 and CLIP2 proteins both contain a typical clip domain and a trypsin-like serine protease domain. Phylogenetic analyses revealed that CLIP1 and CLIP2 belong to subfamily C CLIPs. The qPCR analyses confirmed that both *LsCLIP*1 and *LsCLIP*2 were expressed throughout the developmental stages and in all the assayed larval tissues of *L. serricorne*, with particularly high expression level in the pupal stage and the cuticle, respectively. Expression of both genes was significantly up-regulated in larvae exposed to 20E and following challenge with peptidoglycan from *E. coli* or *S. aureus*.【Conclusion】It is suggested that *LsCLIP*1 and *LsCLIP*2 might be involved in insect molting and innate immune response. These results provide valuable information for further study on the regulation of CLIPs in insects.

Key words: *Lasioderma serricorne*; clip-domain serine proteases; 20-hydroxyecdysone; immune challenge; expression patterns

1 INTRODUCTION

Serine proteases (SPs), encoded by a large and diverse family of genes, are characterized by an active site containing a His-Asp-Ser catalytic triad (Lenz *et al*., 1991; Gatehouse *et al*., 1997). Many studies have demonstrated that SPs participate in various physiological activities, including digestion,

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作者简介：陈春旭，男，1993 年生，贵州遵义人，硕士研究生，研究方向为昆虫生态与害虫综合治理，E-mail：chenchunxu17@163.com

* 通讯作者 Corresponding author, E-mail: axyridis@163.com

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innate immunity, and development (Krem and Cera, 2002; Skernmauritzen *et al.*, 2009). To date, SPs have been identified in a diverse array of organisms, including both vertebrates and invertebrates, and are mainly synthesized as inactive zymogens before being activated by proteolytic cleavage at a specific peptide bond (Yang *et al.*, 2017). Based on their protein structures, SPs are categorized as single-domain and multiple-domain proteins (Ross *et al.*, 2003; Hu *et al.*, 2018). Single-domain SPs are less than 300 amino acid residues in length, and most are putative digestive enzymes. The multiple-domain SPs contain one or more regions for protein-protein interactions, including clip, Sushi, Wonton, scavenger receptor cystine-rich, and other domains, and mainly function in molecular recognition in various biological processes (Cao *et al.*, 2015; Veillard *et al.*, 2016).

Clip-domain serine proteases (CLIPs) contain one or more clip domain(s) at the amino terminus. Each clip domain usually consists of 37 – 55 amino acid residues, including six strictly conserved cysteine residues arranged in three disulfide-bridge structures (Jiang and Kanost, 2000). The first CLIP to be described was purified from hemolymph coagulate from the horseshoe crab, *Tachypleus tridentatus* (Muta *et al.*, 1990). Since then, *CLIP* genes have been identified in numerous insect species. For instance, whole genome sequence analysis has revealed 63, 18, 17, 37, 42, 13, and 30 *CLIP* sequences in *Aedes aegypti* (Brackney *et al.*, 2010), *Apis mellifera* (Zou *et al.*, 2005), *Bombyx mori* (Zhao *et al.*, 2010), *Drosophila melanogaster* (Ross *et al.*, 2003), *Manduca sexta* (Cao *et al.*, 2015), *Plutella xylostella* (Lin *et al.*, 2015), and *Tribolium castaneum* (Consortium *et al.*, 2008), respectively. Based on their phylogenetic relationships, insect CLIPs have been classified into four distinct clades: CLIPA, CLIPB, CLIPC, and CLIPD (Kanost and Jiang, 2015). As one of the most important components of extracellular signaling cascades, CLIPs are involved in insect development and innate immunity. In *D. melanogaster*, two CLIPs (Snake and Easter) were associated with establishing the dorsal-ventral axis of developing embryos (Anderson, 1987; Morisato and Anderson, 1994), while CLIP CG9372 plays a vital role in neuronal projection (Sepp *et al.*, 2008), and Stubble is closely associated with cytoskeleton organization and possibly with wing disc development (Appel *et al.*, 1993; Ibrahim *et al.*, 2013). Recently, RNA interference (RNAi) experiments in *B. mori* demonstrated that knockdown of a typical *CLIP* gene, *BmSP*95, affected cuticle remodeling and led to delayed molting from pupa to adult (Liu *et al.*, 2017). In addition, many studies have shown that CLIPs participate in the immune response of insects, with roles in Toll pathway initiation and prophenoloxidase activation (Kellenberger *et al.*, 2011; Wang *et al.*, 2018). In *M. sexta*, CLIPs were implicated in the stimulation of melanization and in inducing antimicrobial peptide synthesis, with their activities regulated by serpin inhibitors in hemolymph plasma (Wang and Jiang, 2010; An *et al.*, 2013; Yang *et al.*, 2017). In addition, the expression of five CLIP-encoding genes (*PpcSP*6, *PpcSP*9, *PpcSPH*1, *PpcSPH*8, and *PpcSP*74) was significantly up-regulated in *Pteromalus puparum* following infection with different microbial pathogens (Yang *et al.*, 2017).

The cigarette beetle, *Lasioderma serricorne* (Fabricius) (Coleoptera: Anobiidae), is one of the most destructive storage pests in tropical and subtropical regions worldwide (Ashworth, 1993). Infestations of this species affect postharvest stored products in warehouses, including tobacco, grains, spices, traditional Chinese medicine materials, and cereals (Mahroof and Phillips, 2008; Li *et al.*, 2009). Female adults of *L. serricorne* oviposit in the stored products where the larvae develop, causing the consumption of materials and the decline of quality (Kim *et al.*, 2003). Owing to the severe economic impacts of *L. serricorne*, many different pest control methods have been developed, including the use of CO_2-enriched atmosphere (Li *et al.*, 2009), elevated temperature (Yu *et al.*, 2011), low temperature (Imai and Harada, 2006; Collins and Conyers, 2010), and chemical insecticides (Wu *et al.*, 2015; Zhang *et al.*, 2016). However, the high fecundity and adaptability of *L. serricorne* have made it difficult to be controlled using traditional methods. Therefore, new control strategies are urgently needed. We believe that the study of CLIPs in *L. serricorne* may provide new avenues for the control of this important pest.

In this study, we cloned the full-length open reading frames (ORFs) of two CLIPs genes (*LsCLIP*1 and *LsCLIP*2) from *L. serricorne* and examined their expression patterns during different developmental stages and in different tissues of the 5th instar larvae. The effects of exogenous hormone 20-hydroxyecdysone (20E) on the expression of the *LsCLIP* genes in the 4th instar larvae and the responses of *LsCLIP*1 and *LsCLIP*2 to peptidoglycan immune challenges were also investigated. Our findings provide insight and foundation for further functional studies of CLIP-family in insects.

2 MATERIALS AND METHODS

2.1 Insect rearing and sample preparation

The stock colony of *L. serricorne* was originally collected from Guizhou Province, China, and was maintained in the insectary at 28 ± 1℃, 40% ± 5% relative humidity, in darkness. Insects were reared as previously described (Yang *et al.*, 2018).

Samples collected from beetles at the early larval (EL, < 24 h post egg hatching), late larval (LL, older than the 4th instar), pupal (PU, > 48 h post pupation), early adult (EA, < 24 h post eclosion), and late adult (LA, 7 d post eclosion) stages were immediately frozen in liquid nitrogen and then stored at − 80℃. At least 20 insects were randomly collected as a biological replicate, with a total of three independent biological replicates. In the tissue-specific expression experiment, various tissues, including cuticle, fat body, gut, and carcass were isolated from the 5th instar larvae. The larvae were dissected on ice under a stereomicroscope (Olympus SZX12, Tokyo, Japan) to keep tissues fresh, with each sample immediately placed in a 1.5-mL centrifuge tube containing RNA Storage Reagent (Tiangen, Beijing, China). All tissue samples were immediately frozen in liquid nitrogen and stored at − 80℃. At least 30 larvae were collected per replicate, with a total of three independent biological replicates used.

2.2 20E treatment and immune challenge

For 20E experiments, the 4th instar larvae were divided into two groups and treated via microinjection application, as previously reported (Yang *et al.*, 2018). Briefly, a 10 μg/μL stock solution of 20E dissolved in 95% ethanol was diluted to 1 μg/μL with distilled water and used as the working solution. In the 20E treatment group larvae were injected with 20E at a dose of 120 ng/larva using a Nanoliter 2010 injector (World Precision Instruments, Sarasota, FL, USA), while in the control group larvae were injected with an equivalent volume of distilled water containing 0.1% ethanol. Treated individuals were randomly selected from each group at 4, 8, and 12 h post injection for sample collection, with samples frozen in liquid nitrogen and stored at − 80℃. Each treatment group included more than 30 individuals, and the experiment was repeated three times. For the immune challenge, peptidoglycan from *Staphylococcus aureus* (PGN-SA) (InvivoGen, San Diego, CA, USA) and from *Escherichia coli* 0111: B4 (PGN-EB) (InvivoGen, San Diego, CA, USA) were diluted in sterile endotoxin-free water to a final concentration of 1.0 μg/μL. The 4th instar larvae were placed in a Petri dish without food for 6 h for later treatment, and were independently injected with 0.2 μL of PGN-SA solution, PGN-EB solution, or sterile endotoxin-free water (the negative control group). In the blank control group larvae were pricked with the microinjection needle but not subjected to injection. Thirty individual insects were randomly selected from each group at 3, 6, and 9 h post injection for sample collection. All samples were frozen in liquid nitrogen and stored at − 80℃. The experiment was performed in triplicate.

2.3 RNA extraction and cDNA synthesis

Total RNA was isolated from each sample using a MiniBEST Universal RNA Extraction Kit (TaKaRa, Dalian, China), including gDNA Eraser Spin Column for genomic DNA removal. The quality and purity of extracted RNA were measured at 260 and 280 nm using a NanoDrop 2000C Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and the integrity was further verified by 1.0% agarose gel electrophoresis. First strand cDNA synthesis was performed from 1 μg of total RNA using a PrimeScript RT Reagent Kit (TaKaRa) with random hexamers and oligo (dT) primers.

2.4 Molecular cloning of two *LsCLIP* genes

Based on the transcriptomic data of *L. serricorne* (unpublished data), two pairs of specific primers were designed to amplify the full-length ORFs of two *LsCLIP* genes by reverse-transcription polymerase chain reaction (RT-PCR). PCR parameters were 95℃ for 3 min, followed by 35 cycles of 95℃ for 30 s, 58℃ for 30 s, and 72℃ for 1.5 min, with a final extension step of 72℃ for 10 min. All PCR products were separated by 1% agarose gel electrophoresis and sub-cloned into a pGEM-T Easy vector (Promega, Madison, WI, USA) for sequencing.

2.5 Bioinformatic and phylogenetic analyses

The ORFs were predicted using ORF Finder software (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) and the deduced amino acid sequences were aligned using DNAMAN 6.0 (LynnonBiosoft, Quebec, Canada). The signal peptide was predicted using SingnalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/). The simple modular architecture research tool (SMART; http:/smart.embl.de/) was used to predict conserved domains. The molecular weight and isoelectric point were analyzed using ExPASy (http://web.expasy.org/compute_pi/). A phylogenetic tree based on insect CLIPs sequences was constructed using MEGA 6.06 (Tamura *et al.*, 2013) by the neighbor-joining method with 1 000 bootstrap replicates and pairwise deletion.

2.6 Quantitative RT-PCR (qPCR)

qPCR analyses were performed using the CFX-96 real-time PCR system (Bio-Rad, Hercules, CA, USA) in a total reaction volume of 20 μL containing 10 μL of GoTaq qPCR Master Mix (Promega), 1 μL of cDNA template (500 ng/μL), 1 μL of each gene-specific primer (10 μmol/L), and 7 μL of nuclease-free water. All primers used for qPCR were designed using Primer 3 (version 0.4.0) software (http://fokker.wi.mit.edu/primer3/) and were listed in Table 1. The reaction conditions were: 94℃ for 3 min, followed by 40 cycles of 94℃ for 30 s and 60℃ for 30 s. At the end of the cycling protocol, a melting curve from 60 to 95℃ was applied to verify the production of single PCR products. All experiments were performed in triplicate, with two technical replicates. The 18S rRNA gene (GenBank accession no.: MK033476) was used as an internal reference gene. The relative expression levels of the two *LsCLIP* genes were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.7 Data analysis

SPSS 17.0 software (IBM Corp, Chicago, IL, USA) was used for statistical analysis. Expression data were presented as the mean ± *SE* (standard error). Significant differences were analyzed by one-way analysis of variance (ANOVA) followed by a least significant difference (LSD) test (for multiple comparisons) or a two-tailed, unpaired *t*-test (for comparison of two means).

Table 1 Primers used in this study

Primers	Primer sequences (5′-3′)	Product size (bp)	Use of primers
LsCLIP1-F	GGGAAACATGGGATTAGTTGCT	1 206	Full-length confirmation
LsCLIP1-R	TGAACTCAAGGCCACACTATCCT		
LsCLIP2-F	AGTGAAGAATGCAGTCTAGC	1 211	
LsCLIP2-R	TATGGACGTTCAATTGGACG		
Q-LsCLIP1-F	CGACAGGAACGTACAGAGCA	162	qPCR analysis
Q-LsCLIP1-R	ATCGACACCGGATGTATGGT		
Q-LsCLIP2-F	TGGAACACGTGGTGAACACT	221	
Q-LsCLIP2-R	GTCCATCCTCTCGTCTCTGC		
Q-18S-F	GTTGATCACGTCGCAAGCTA	208	
Q-18S-R	AGGTTTCCCTCTGGCTTGTT		

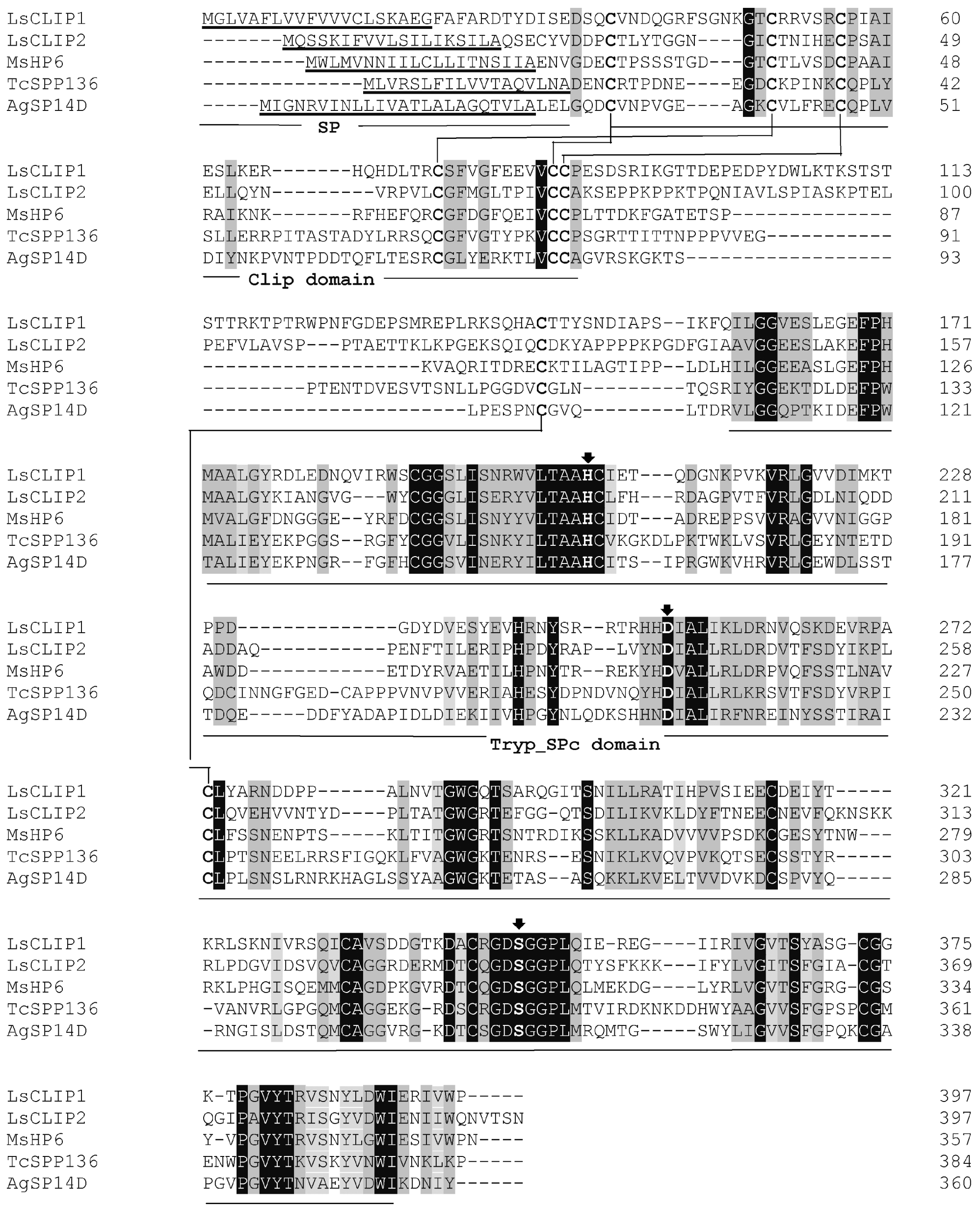
3 RESULTS

3.1 Identification and sequencing analysis of two *LsCLIP* genes

Based on the *L. serricorne* transcriptome, two distinct cDNA fragments putatively encoding CLIP proteins were identified by keyword searching. We subsequently cloned the full-length ORFs of two *LsCLIP* genes (*LsCLIP*1 and *LsCLIP*2) by RT-PCR, and deposited the sequences in the GenBank database under accession numbers MK015723 (*LsCLIP*1) and MK015722 (*LsCLIP*2). The ORFs of the two *LsCLIP* genes are both 1 194 bp in length, encoding 397 amino acid residues. The calculated molecular weights of LsCLIP1 and LsCLIP2 are 44.3 and 43.4 kD, and pI values are 6.18 and 5.19, respectively. Signal peptides of 20 and 19 amino acids were identified in the N-terminal regions of the deduced LsCLIP1 and LsCLIP2 proteins, respectively, indicating that both LsCLIPs are secreted proteins. Domain analysis showed that both proteases contain a clip domain and a conserved trypsin-like serine protease domain (Tryp_SPc domain) at the C-terminal region, with six cysteine residues forming three disulfide linkages found in both clip domains (Fig. 1). Multiple alignment of CLIP amino acid sequences from *L. serricorn*, *M. sexta*, *T. castaneum*, and *Anopheles gambiae* conducted using DNAMAN showed that these sequences share high homology, with several conserved motifs including six cysteine residues in the clip domain, the catalytic His-Asp-Ser triad, and the cysteine residues in the Tryp_SPc domain (Fig. 1).

3.2 Phylogenetic analysis of LsCLIPs

A phylogenetic tree was constructed using the amino acid sequences of 56 CLIPs from seven insect species. The sequences clustered into each of the four subfamilies: CLIPA, CLIPB, CLIPC, and CLIPD. While LsCLIP1 formed a cluster with MsHP6 from *M. sexta*, LsCLIP2 showed a close relationship with AgCLIP 1 and AgCLIP 3 from *A.*



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LsCLIP1    MGLVAFLVVFVVVCLSKAEGFAFARDTYDISEDSQCVNDQGRFSGNKGTCRRVSRCPIAI   60
LsCLIP2    -------MQSSKIFVVLSILIKSILAQSECYVDDPCTLYTGGN----GICTNIHECPSAI   49
MsHP6      ---------MWLMVNNIILCLLITNSIIAENVGDECTPSSSTGD---GTCTLVSDCPAAI   48
TcSPP136   --------------MLVRSLFILVVTAQVLNADENCRTPDNE----EGDCKPINKCQPLY   42
AgSP14D    -----MIGNRVINLLIVATLALAGQTVLALELGQDCVNPVGE----AGKCVLFRECQPLV   51

LsCLIP1    ESLKER-------HQHDLTRCSFVGFEEVVCCPESDSRIKGTTDEPEDPYDWLKTKSTST   113
LsCLIP2    ELLQYN---------VRPVLCGFMGLTPIVCCAKSEPPKPPKTPQNIAVLSPIASKPTEL   100
MsHP6      RAIKNK-------RFHEFQRCGFDGFQEIVCCPLTTDKFGATETSP--------------   87
TcSPP136   SLLERRPITASTADYLRRSQCGFVGTYPKVCCPSGRTTITTNPPPVVEG-----------   91
AgSP14D    DIYNKPVNTPDDTQFLTESRCGLYERKTLVCCAGVRSKGKTS------------------   93

LsCLIP1    STTRKTPTRWPNFGDEPSMREPLRKSQHACTTYSNDIAPS--IKFQILGGVESLEGEFPH   171
LsCLIP2    PEFVLAVSP---PTAETTKLKPGEKSQIQCDKYAPPPPKPGDFGIAAVGGEESLAKEFPH   157
MsHP6      -------------------KVAQRITDRECKTILAGTIPP--LDLHILGGEEASLGEFPH   126
TcSPP136   ---------PTENTDVESVTSNLLPGGDVCGLN---------TQSRIYGGEKTDLDEFPW   133
AgSP14D    -----------------------LPESPNCGVQ---------LTDRVLGGQPTKIDEFPW   121

LsCLIP1    MAALGYRDLEDNQVIRWSCGGSLISNRWVLTAAHCIET---QDGNKPVKVRLGVVDIMKT   228
LsCLIP2    MAALGYKIANGVG---WYCGGGLISERYVLTAAHCLFH---RDAGPVTFVRLGDLNIQDD   211
MsHP6      MVALGFDNGGGE--YRFDCGGSLISNYYVLTAAHCIDT---ADREPPSVVRAGVVNIGGP   181
TcSPP136   MALIEYEKPGGS--RGFYCGGVLISNKYILTAAHCVKGKDLPKTWKLVSVRLGEYNTETD   191
AgSP14D    TALIEYEKPNGR--FGFHCGGSVINERYILTAAHCITS--IPRGWKVHRVRLGEWDLSST   177

LsCLIP1    PPD--------------GDYDVESYEVHRNYSR--RTRHHDIALIKLDRNVQSKDEVRPA   272
LsCLIP2    ADDAQ------------PENFTILERIPHPDYRAP--LVYNDIALLRLDRDVTFSDYIKPL   258
MsHP6      AWDD------------ETDYRVAETILHPNYTR--REKYHDVALLRLDRPVQFSSTLNAV   227
TcSPP136   QDCINNGFGED-CAPPPVNVPVVERIAHESYDPNDVNQYHDIALLRLKRSVTFSDYVRPI   250
AgSP14D    TDQE-----DDFYADAPIDLDIEKIIVHPGYNLQDKSHHNDIALIRFNREINYSSTIRAI   232

LsCLIP1    CLYARNDDPP------ALNVTGWGQTSARQGITSNILLRATIHPVSIEECDEIYT-----   321
LsCLIP2    CLQVEHVVNTYD----PLTATGWGRTEFGG-QTSDILIKVKLDYFTNEECNEVFQKNSKK   313
MsHP6      CLFSSNENPTS-----KLTITGWGRTSNTRDIKSSKLLKADVVVVPSDKCGESYTNW---   279
TcSPP136   CLPTSNEELRRSFIGQKLFVAGWGKTENRS--ESNIKLKVQVPVKQTSECSSTYR-----   303
AgSP14D    CLPLSNSLRNRKHAGLSSYAAGWGKTETAS--ASQKKLKVELTVVDVKDCSPVYQ-----   285

LsCLIP1    KRLSKNIVRSQICAVSDDGTKDACRGDSGGPLQIE-REG----IIRIVGVTSYASG-CGG   375
LsCLIP2    RLPDGVIDSVQVCAGGRDERMDTCQGDSGGPLQTYSFKKK---IFYLVGITSFGIA-CGT   369
MsHP6      RKLPHGISQEMMCAGDPKGVRDTCQGDSGGPLQLMEKDG----LYRLVGVTSFGRG-CGS   334
TcSPP136   -VANVRLGPGQMCAGGEKG-RDSCRGDSGGPLMTVIRDKNKDDHWYAAGVVSFGPSPCGM   361
AgSP14D    -RNGISLDSTQMCAGGVRG-KDTCSGDSGGPLMRQMTG-----SWYLIGVVSFGPQKCGA   338

LsCLIP1    K-TPGVYTRVSNYLDWIERIVWP-----   397
LsCLIP2    QGIPAVYTRISGYVDWIENIIWQNVTSN   397
MsHP6      Y-VPGVYTRVSNYLGWIESIVWPN----   357
TcSPP136   ENWPGVYTKVSKYVNWIVNKLKP-----   384
AgSP14D    PGVPGVYTNVAEYVDWIKDNIY------   360
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Fig. 1　Multiple sequence alignment of the two deduced LsCLIP proteins from *Lasioderma serricorne* with CLIP proteins from other insects

Amino acid sequences were obtained from the GenBank database for CLIP proteins from *Manduca sexta* (MsHP6, GenBank accession no.: AAV91004), *Tribolium castanum* (TcSPP136, GenBank accession no.: EFA07558), and *Anopheles gambiae* (AgSP14D, GenBank accession no.: AAB62929). Identical and similar residues are shaded. The predicted signal peptide (SP), clip domain, and trypsin-like serine protease (Tryp_SPc) domain are underlined. The conserved disulfide bonds are labeled with connecting lines. The residues forming the catalytic triad (His, Asp, and Ser) are marked with an arrow.

gambiae; however, both proteins were grouped into the CLIPC subfamily (Fig. 2).

3.3　Temporal and spatial expression profiles of *LsCLIP*1 and *LsCLIP*2 in *L. serricorne*

To further study the function of the two *LsCLIPs* genes, we examined their developmental expression patterns in *L. serricorne*. The results showed that both genes were expressed across all the assayed developmental stages [early larva (< 24 h post egg hatching), late larva (older than the 4th instar), pupa (> 48 h post pupation), early adult (< 24 h post eclosion) and late adult (7 d post eclosion)], and that their expression levels varied significantly throughout the three developmental stages. *LsCLIP*1 and *LsCLIP*2 had a similar expression patterns, with the highest expression levels during the pupal stage, and the lowest expression levels at the late adult stage (Fig. 3).

qPCR analysis also showed that both *LsCLIP* genes were expressed in all the assayed tissues (cuticle, fat body, gut and carcass) of the 5th instar larvae of *L. serricorne*, with particularly high

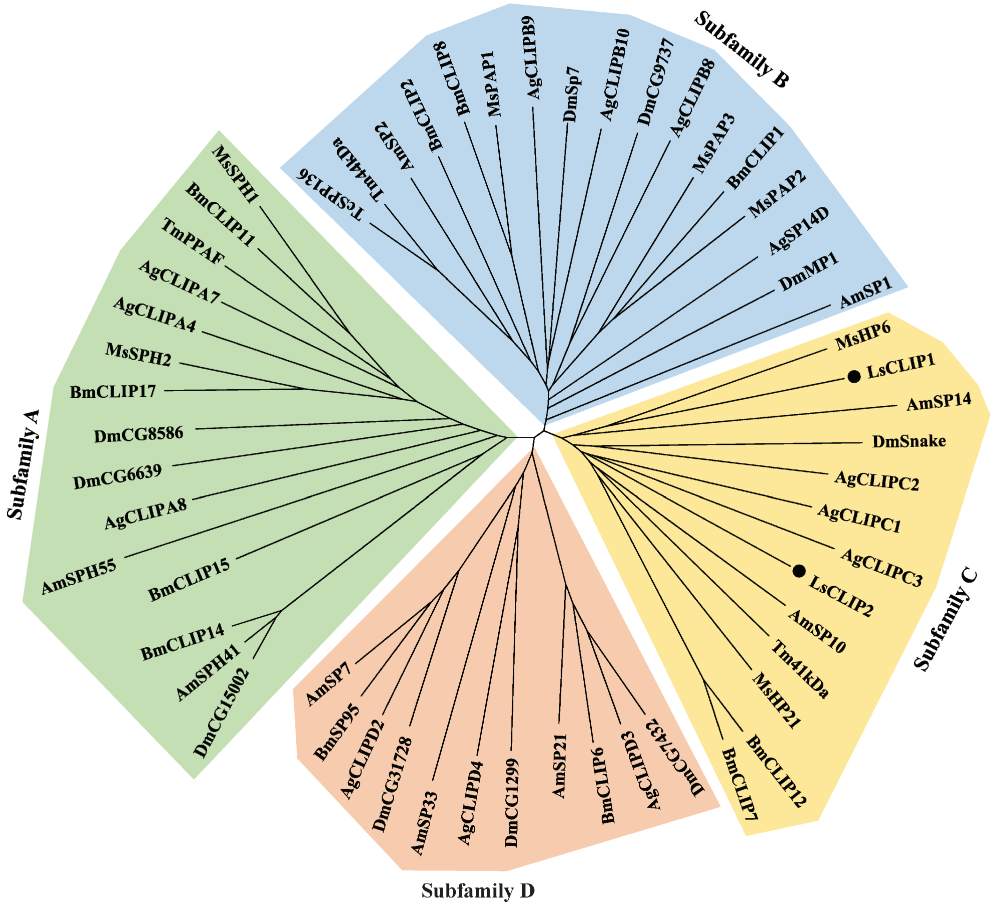

Fig. 2 Phylogenetic analysis of CLIPs from *Lasioderma serricorne* and other insects

The neighbor-joining tree was constructed using MEGA 6.06. Numbers above each branch are bootstrap support values calculated from 1 000 replicates. The following GenBank sequences were used: *Bombyx mori* BmCLIP1 (NP_001036832), BmCLIP2 (NP_001036844), BmCLIP17 (NP_001040462), BmCLIP11 (NP_001037053), BmCLIP15 (XP_004931455), BmCLIP8 (XP_012548716), BmCLIP12 (XP_004927620), BmCLIP7 (XP_004926962), BmSP95 (XP_012549295), BmCLIP14 (XP_004925813), BmCLIP6 (XP_012545524); *Drosophila melanogaster* DmCG6639 (AAF53614), DmCG8586 (AAF59059), DmCG15002 (AAF47850), DmCG9737 (AAF57029), DmSp7 (AAF54143), DmMP1 (AAF52151), DmSnake (AAF54897), DmCG1299 (AAF47847), DmCG31728 (AAF53273), DmCG7432 (AAF55692); *Anopheles gambiae* AgCLIPA4 (XP_552464), AgCLIPA7 (XP_320723), AgCLIPA8 (XP_311445), AgCLIPB8 (XP_312743), AgCLIPB9 (XP_003436374), AgCLIPB10 (XP_312744), AgCLIPC1 (XP_552698), AgCLIPC2 (XP_313588), AgCLIPC3 (XP_313589), AgCLIPD4 (XP_312102), AgCLIPD2 (XP_317284), AgCLIPD3 (XP_321698), AgSP14D (AAB62929.1); *Tribolium castaneum* TcSPP136 (EFA07558.1); *Tenebrio molitor* TmPPAF (CAC12696.1), Tm41kDa (BAG14261.1), Tm44kDa (BAG14262.2); *Apis mellifera* AmSPH41 (XP_006563756), AmSPH55 (XP_001120817), AmSP2 (XM_006570203), AmSP1 (XP_006570267), AmSP10 (XP_001120043), AmSP14 (XP_001121032), AmSP7 (XP_625051), AmSP21 (XP_006567247), AmSP33 (XP_006559393); *Manduca sexta* MsHP6 (AAV91004.1), MsHP21 (AAV91019.1), MsSPH2 (AF518768_1), MsSPH1 (AAM69352.2), MsPAP3 (AAO74570.1), MsPAP2 (AAL76085.1), MsPAP1 (AAX18636.1); *Lasioderma serricorne* LsCLIP1 (MK015723), LsCLIP2 (MK015722).

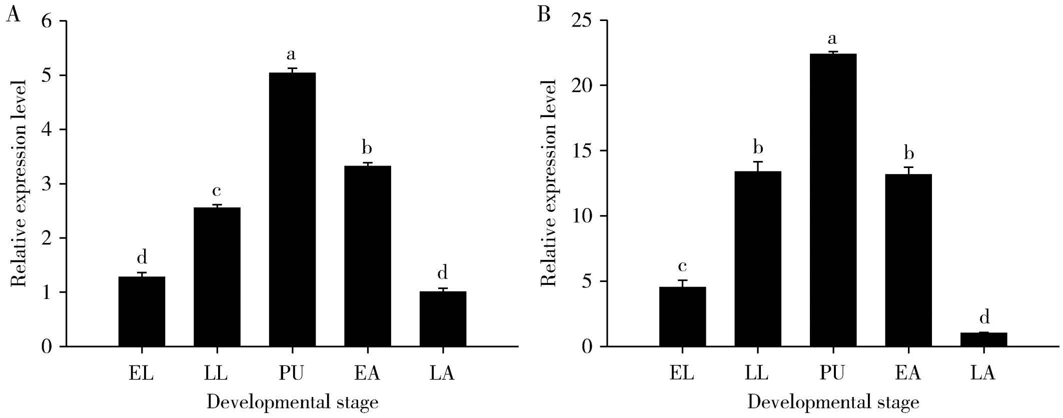

Fig. 3 Relative expression levels of *LsCLIP*1 (A) and *LsCLIP*2 (B) in *Lasioderma serricorne* at different developmental stages

EL: Early larva (< 24 h post egg hatching); LL: Late larva (older than the 4th instar); PU: Pupa (> 48 h post pupation); EA: Early adult (< 24 h post eclosion); LA: Late adult (7 d post eclosion). Total insect bodies were collected for qPCR analysis. The lowercase letters on the bars indicate significant differences among developmental stages based on one-way ANOVA followed by a least significant difference test ($P < 0.05$).

expression level in the cuticle and low expression level in the gut. Both *LsCLIP*1 and *LsCLIP*2 transcripts were also abundant in the fat body and carcass (Fig. 4).

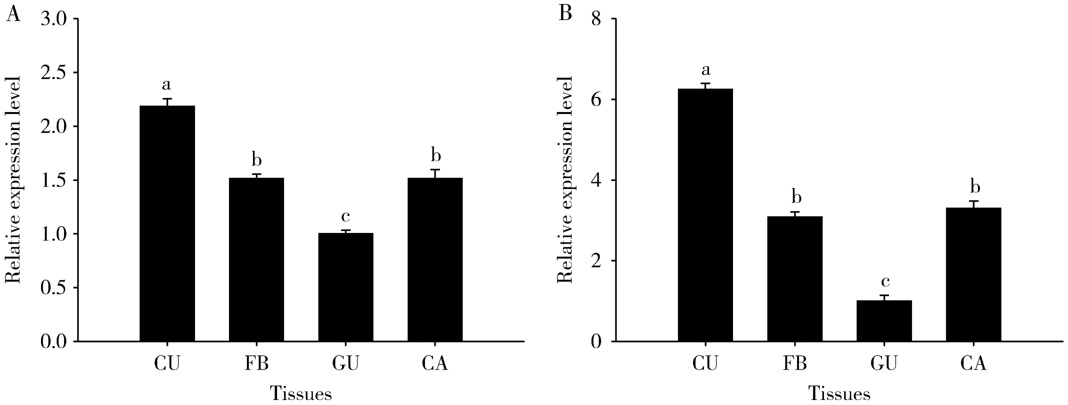

Fig. 4 Relative expression levels of *LsCLIP*1 (A) and *LsCLIP*2 (B) in different tissues of the 5th instar larvae of *Lasioderma serricorne*

CU: Cuticle; FB: Fat body; GU: Gut; CA: Carcass. Different letters above bars indicate significant differences among tissues based on one-way ANOVA followed by a least significant difference test ($P<0.05$).

3.4 Relative expression levels of *LsCLIP*1 and *LsCLIP*2 in the 4th instar larvae of *L. serricorne* following 20E treatment

qPCR analysis was also carried out to determine the effect of 20E exposure on the mRNA levels of the two *LsCLIP* genes. Compared with the control, 20E treatment significantly enhanced the mRNA levels of both *LsCLIP* genes in the 4th instar larvae of *L. serricorne*. Their expression peaks occurred at 8 h post injection, with a 13.7-fold increase in the expression level of *LsCLIP*1 (Fig. 5: A) and a 43.5-fold increase in the expression level of *LsCLIP*2 (Fig. 5: B) observed at this time point.

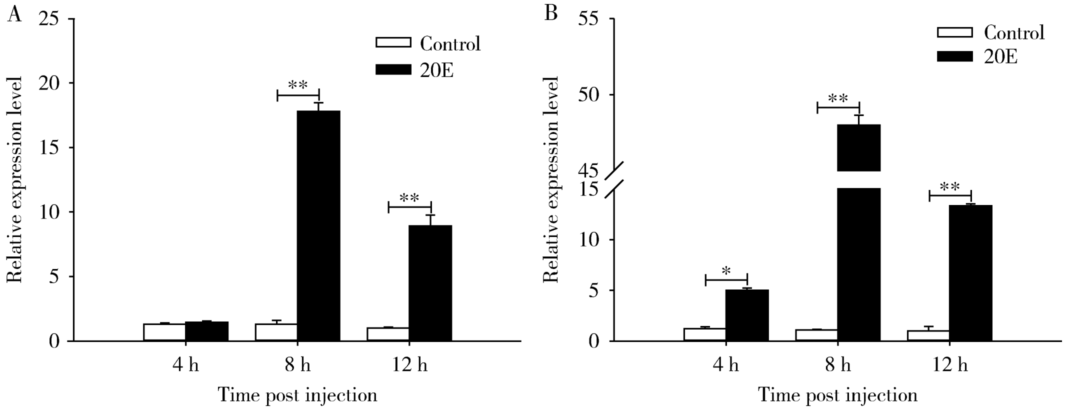

Fig. 5 Relative expression levels of *LsCLIP*1 (A) and *LsCLIP*2 (B) in the 4th instar larvae of *Lasioderma serricorne* in response to 20E induction

Total insect bodies of larvae were collected for qPCR analysis at 4, 8 and 12 h post injection with 20E. In the control group, larvae were injected with distilled water containing 0.1% ethanol, while in the 20E treatment group, larvae were injected with 20E (120 ng/larva). Significant differences between the treatment group and the control group at the same point were determined using a two-tailed, unpaired *t*-test and indicated by asterisk ($P<0.05$) or double asterisk ($P<0.01$).

3.5 Transcriptional response of *LsCLIP*1 and *LsCLIP*2 in the 4th instar larvae of *L. serricorne* to immune challenge

To examine whether *LsCLIP*1 and *LsCLIP*2 are immune-responsive genes in *L. serricorne*, the transcript levels of both genes in the 4th instar larvae following PGN-EB or PGN-SA injection were detected by qPCR. *LsCLIP*1 transcript levels were significantly up-regulated at 3 and 6 h post PGN-SA injection, and then dropped at 9 h post injection. The expression of *LsCLIP*1 was remarkably up-regulated and peaked at 9 h post PGN-EB challenge and 6 h post PGN-SA challenge (Fig. 6: A). The relative expression level of *LsCLIP*2 was also significantly up-regulated at 3 – 9 h post challenge with PGN-EB, with the ratios of inducible expression to constitutive expression of *LsCLIP*2 calculated as 8.6-, 8.6-, and 4.3-fold (compared with expression levels in larvae injected with water) at 3, 6, and 9 h post treatment, respectively (Fig. 6: B). The relative mRNA levels of *LsCLIP*2 were significantly increased at 3 and 6 h post PGN-SA injection; however, that of *LsCLIP*2 showed no significant increase at 9 h post treatment.

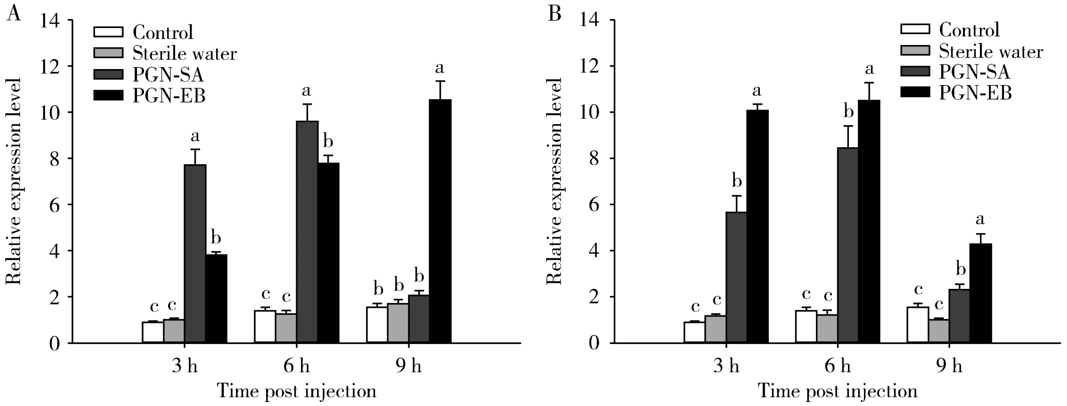

Fig. 6 Relative expression levels of *LsCLIP*1 (A) and *LsCLIP*2 (B) in the 4th instar larvae of *Lasioderma serricorne* following challenge with PGN-SA or PGN-EB

Total insect bodies of larvae were collected for qPCR analysis at 3, 6 and 9 h post injection of peptidoglycans. In the blank control group, larvae were pricked with the microinjection needle but not subjected to injection; in the sterile water group (the negative control group), larvae were injected with 0.2 μL of sterile endotoxin-free water; in the PGN-SA treatment group larvae were injected with 0.2 μL of PGN-SA solution; and in the PGN-EB treatment group larvae were injected with 0.2 μL of PGN-EB solution. Different letters above bars indicate significant differences based on one-way ANOVA followed by a least significant difference test ($P < 0.05$).

4 DISCUSSION AND CONCLUSION

Because of their pivotal roles in insect development and immune processes, CLIPs have received much attention and are regarded as potential targets for pest control (Kanost *et al*., 2001, 2004). Since the first *CLIP* gene was identified in *D. melanogaster* (Morisato and Anderson, 1994), an increasing number of *CLIPs* from several insect species have been documented. CLIPs can be divided into four subfamilies (A - D), based on bioinformatic analyses. CLIPC is a major subfamily and has been relatively well studied in insects. CLIPC proteases have a type 1a clip domain and mainly function in innate immunity. They also activate CLIPB proteases to activate immune effector proteins or cytokines (Kanost and Jiang, 2015). In this study, two full-length cDNAs corresponding to CLIP-encoding genes *LsCLIP*1 and *LsCLIP*2 were identified and cloned from the cigarette beetle *L. serricorne*. Multiple sequence alignment revealed that, the two corresponding LsCLIP proteins were similar to those reported in other insect species. A conserved clip domain, which acts in protein-protein interactions and in the regulation of antimicrobial and protease activities (Jiang and Kanost, 2000), was located in both predicted LsCLIP amino acid sequences. The presence of classical signal peptides suggests that the two LsCLIP proteins are likely to be secreted proteins, which is consistent with other CLIPs such as ApSnake from *Antheraea pernyi* and MsHP6 from *M. sexta*. A Tryp_SPc domain at the C-terminus, which is responsible for folding chymotrypsin (Kanost and Jiang, 2015), is highly conserved among all the examined amino acid sequences (Fig. 1). Phylogenetic analysis showed that the two LsCLIPs and their homologs clustered within the CLIPC subfamily (Fig. 2). The high level of conservation of structural domains and the conserved phylogenetic relationships suggest that CLIPs carry out important biological functions in insects.

Developmental expression profiles (Fig. 3) revealed that *LsCLIP*1 and *LsCLIP*2 had high expression levels in the late larval and pupal stages, which are the key periods for larval growth and pupal formation. It is thought that *LsCLIPs* might play an important role in regulating the molting processes of *L. serricorne*. Similar observations have been made in *B. mori*, where the expression of *BmSP*95 peaks during each molting period then sharply decreases during the intermolt periods (Liu *et al*., 2017). In *A. pernyi*, high levels of *ApSnake* transcript were detected in the 4th and 5th instar larvae (Wang *et al*., 2018). CLIP-encoding gene *snake* 4 is mainly expressed in the 5th instar nymphs of *Nilaparvata lugens* (Bao *et al*., 2014). Western blot analyses indicated that CLIP PrSP1 is mainly expressed in the plasma of *Pieris rapae* (Zhu *et al*., 2011). In endoparasitoid *P. puparum*, *CLIP* genes are mainly expressed during the larval stage, when the wasps mature inside their hosts and need to generate abundant CLIP proteases to resist environmental stresses (Yang *et al.*, 2017). Additionally, *LsCLIP*1 and *LsCLIP*2 showed the highest expression levels in the pupal stage (Fig. 3). Similar expression patterns have also been observed in antimicrobial peptide genes in other species. For example, *defensin* in *B. mori* (Wen *et al.*, 2009), and *lebocin* in *Spodoptera litura* (Mai *et al.*, 2017). Thus, we presume that these two LsCLIPs might be involved in immune defense process during the pupal stage of *L. serricorne*.

In numerous studies, CLIP expression has been noted in multiple insect tissues, with the different tissue-specific expression patterns reflecting the diverse biological functions of the proteins. In the present study, both of the two *LsCLIP* genes were widely expressed in all the assayed larval tissues (Fig. 4), indicating their potential multifunctionality. The highest levels of gene expression were observed in the cuticle, which is consistent with previous studies in *B. mori* (Liu *et al.*, 2017) and *Octodonta nipae* (Zhang *et al.*, 2017). The cuticle is an important organ for insect molting and metamorphosis, while it is the first line of defense against microbial infection. Many insect-based studies have reported that CLIP transcripts are primarily distributed in immune-related organs such as the fat body or hemolymph, including in *Tenebrio molitor* (Lee *et al.*, 2002), *O. nipae* (Zhang *et al.*, 2017), and *Acromyrmex echinatior* (Baer *et al.*, 2005). Interestingly, *MsHP*17 is normally not detectable in the fat body or hemolymph of *M. sexta*, but its expression levels increase in both tissues following microbiol infection (Jiang *et al.*, 2005). The higher levels of expression of the two *LsCLIP* genes in the fat body in the current study may relate to their vital roles in immune response (Jiang *et al.*, 2005; Zou *et al.*, 2005). In addition, high levels of expression of several *CLIP* genes were specifically detected in the venom gland of *P. puparum*, suggesting that these genes may be involved in parasitism (Yang *et al.*, 2017).

There is a functional connection between 20E and insect CLIP proteases. In *B. mori*, *BmSP*95 was mainly expressed during molting and metamorphosis, which coincides with the fluctuating 20E titer (Kiguchi and Agui, 1981; Liu *et al.*, 2017). The expression of *BmSP*95 was substantially up-regulated following injection of 20E *in vivo*. In the present study, we found that 20E up-regulated the mRNA levels of the two *LsCLIP* genes at 8 and 12 h post treatment (Fig. 5). This expression pattern is consistent with that of β-N-acetylglucosaminidase-encoding gene *LsNAG*1, which is crucial for chitin degradation and larval-pupal transition in *L. serricorne* (Yang *et al.*, 2018). Previous studies have demonstrated that insect innate immunity can be affected by juvenile hormone (JH) and 20E. In *B. mori*, the mRNA levels and activities of some antimicrobial peptides were dramatically increased following JH treatment; however, the induced effects were inhibited by 20E treatment (Tian *et al.*, 2010). Collectively, the results suggest that the two *LsCLIP* genes are important 20E-late-responsive genes and play major roles during the larval-pupal molt.

Insect CLIPs probably have an antimicrobial function as part of the innate immune response (An *et al.*, 2013). In the current study, both PGN-EB and PGN-SA could stimulate the expression of the two *LsCLIP* genes (Fig. 6), indicating that they participate in the host immune response to the different peptidoglycans. PGN-SA and PGN-EB are purified peptidoglycans from Gram-positive bacterium *S. aureus* and Gram-negative bacterium *E. coli*, respectively. Different pathogens stimulate different immune signaling pathways. For example, Gram-positive bacteria, yeast, and fungi can stimulate the Toll pathway, whereas Gram-negative bacteria may stimulate an immune deficiency (IMD) pathway (Amparyup *et al.*, 2010; Hillyer, 2016; Liu *et al.*, 2016). In *D. melanogaster*, *DmMSP* recognized Gram-positive bacterium *S. aureus* via peptidoglycan-recognition molecules and then autoactivated the serine proteinase cascade of the prophenoloxidase system or the Toll signaling pathway (Buchon *et al.*, 2009), while HP14 had the exact same effect in *M. sexta* (Wang and Jiang, 2010, 2017). Biochemical analyses have indicated that CLIPs interact directly with pattern-recognition receptor complexes involved in the sensing of peptidoglycan (Kim *et al.*, 2008; Buchon *et al.*, 2009). Moreover, both melanin synthesis and phenoloxidase activity in *Ostrinia furnacalis* were induced by the injection of entomopathogenic fungus *Beauveria bassiana* (Chu *et al.*, 2015). These results suggested that CLIPs are essential for the activation of the Toll signaling pathway by Gram-positive bacteria and fungi. In comparison, *E. coli* treatment induced *PrSP*1 expression in *P. rapae* and may be involved in the innate immune response (Zhu *et al.*, 2011), while *ApSnake* transcription was also stimulated by *E. coli* in *A. pernyi* (Wang *et al.*, 2018). Furthermore, similar up-regulation of the expression of insect *CLIP* genes following exposure to different pathogens has been reported in species including *B. mori* (Zhao *et al.*, 2010), *O. nipae* (Zhang *et al.*, 2017), and *A. gambiae* (Povelones *et al.*, 2013). In *P. puparum*, the expression of *PpcSPH*1 was increased following treatment with *E. coli*, *Micrococcus luteus*, or *B. bassiana*, suggesting that the corresponding protein might behave as a vital cofactor upstream of the protease cascade (Yang *et al.*, 2017). The up-regulation of the two *LsCLIP* genes following exposure to both Gram-positive and Gram-negative peptidoglycan indicates their involvement in the immune response. However, the specific mechanism by which they help prevent infection remains to be determined. In summary, we identified and characterized two CLIP-encoding genes (*LsCLIP*1 and *LsCLIP*2) in *L. serricorne*. The predicted LsCLIP proteins contain typical domain structures of the CLIPC subfamily. The two genes showed similar expression patterns during different developmental stages and in different larval tissues. Transcription of the two genes was significantly increased after exposure to 20E and the following challenge with PGN-EB or PGN-SA. Interestingly, these two LsCLIPs clustered into the CLIPC subfamily, but showed low sequence similarity. Similar expressions of two *LsCLIPs* indicate that they might have the same function and are most likely involved in insect molting and the innate immune response. This is consistent with other studies, in which five amino acid sequences of trypsin in *Bactrocera dorsalis* were quite different, but all those genes were actively involved in digestion process and larval development (Hou *et al.*, 2014; Li *et al.*, 2017). Additional studies are needed to clarify the physiological mechanism of these two CLIP proteins in *L. serricorne*.

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烟草甲 clip 丝氨酸蛋白酶基因的克隆及其对外源激素和免疫胁迫的表达响应

陈春旭[1,2], 许抗抗[1,2], 杨　洪[1,3,*], 赵　凤[1], 严　毅[1,2], 胡大鸣[4], 杨文佳[1,2]

(1. 贵州大学昆虫研究所, 贵州山地农业病虫害重点实验室, 贵阳 550025;
2. 贵阳学院生物与环境工程学院, 贵州省山地珍稀动物与经济昆虫重点实验室, 贵阳 550005;
3. 贵州大学烟草学院, 贵阳 550025; 4. 贵州中烟工业有限责任公司, 贵阳 550025)

摘要:【目的】作为细胞外信号级联通路的重要组成部分,含有 clip 结构域的丝氨酸蛋白酶(clip-domain serine proteases, CLIPs)在昆虫发育和先天免疫过程中起着重要作用。本研究旨在克隆烟草甲 *Lasioderma serricorne* CLIP 基因,解析其在烟草甲不同发育阶段和幼虫不同组织中的表达模式,分析其在外源激素 20-羟基蜕皮酮(20E)和免疫胁迫后的表达特征,为进一步研究其生理功能奠定基础。【方法】采用 RT-PCR 技术克隆获得烟草甲两个 *CLIPs* 基因(*LsCLIP*1 和 *LsCLIP*2)全长 cDNA 序列,并利用生物信息学软件预测其编码蛋白的结构和特征,利用 MEGA 6.06 构建昆虫 CLIPs 系统发育树;利用实时荧光定量 PCR(quantitative real-time PCR, qPCR)研究这两个基因在不同发育阶段[低龄幼虫(卵孵化后 24 h 内)、高龄幼虫(4 龄以上)、蛹(化蛹后 48 h 以上)、早期成虫(化蛹后 24 h 内)和晚期成虫(化蛹后 7 d)]、5 龄幼虫不同组织(表皮、脂肪体、肠道和剩余组织)中以及注射 20E(120 ng/幼虫)和来源于大肠杆菌 *Escherichia coli* 和金黄色葡萄球菌 *Staphylococcus aureus* 的肽聚糖(0.2 μL)后 4 龄幼虫中的表达模式。【结果】克隆获得烟草甲 *LsCLIP*1 和 *LsCLIP*2 基因的 cDNA 全序列,其开放阅读框长度均为 1 194 bp,编码 397 个氨基酸。序列分析显示,其氨基酸序列各自具有一个 clip 结构域和胰蛋白酶结构域。系统发育分析表明,CLIP1 和 CLIP2 都属于 subfamily C CLIPs。qPCR 结果表明,*LsCLIP*1 和 *LsCLIP*2 基因在所检测的各发育阶段和幼虫各组织中均有表达,分别尤以蛹期和表皮中表达量最高;经 20E 和肽聚糖诱导后,烟草甲幼虫体内 *LsCLIP*1 和 *LsCLIP*2 基因的表达量明显提高。【结论】推测 *LsCLIP*1 和 *LsCLIP*2 可能参与了烟草甲的蜕皮发育和对免疫胁迫的应激响应。本研究将为后续研究昆虫 CLIPs 的分子调控提供参考。

关键词: 烟草甲; clip 丝氨酸蛋白酶; 20-羟基蜕皮甾酮; 免疫胁迫; 表达模式

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